MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERIZATION OF SPECIFIC MICROBIAL STRAINS ISOLATED FROM LIQUID SUBSTRATES

Afriola Ceta¹, Anisa Kristo¹, Klodjan Zotaj¹, Elena Muca¹, Rozana Troja*¹

¹Department of Industrial Chemistry, Faculty of Natural Sciences University of Tirana, Boulevard ZOG I, 1000 Tirana, Albania
*e-mail: rozitroja@yahoo.com

Abstract

Extreme conditions effect the growth of microorganisms. The effect of high percentage of salt and high values also of osmotic pressure, caused by high percentage of sugars in selected samples, have been investigated during the experimental work here presented. So, waters from coastal towns and liquid pharmaceuticals with high percentage of carbon sources were used as important contributors for the isolation and identification of microbial strains with specific morphological and physiological characteristics.

These substrates were considered very important for the isolation of bacterial and yeast colonies including those of osmotolerants, osmophiles and pigmented.

Selected substrates were microbiologically analyzed and screening methods were used for obtaining a great number of bacteria, yeasts and molds. Microbiological evaluation was made in order to determine morphological and physiological characteristics of isolated and purified strains. Microbiological methods of analysis (Wickerham, Lodder and others) were used to observe how special conditions of development affect tendencies of sporulation, pseudo micelle formation, pigmentation and other morphological characteristics. Physiological characterization of isolated and purified cultures, including auxonographic methods of sugar assimilation was performed, in order to complete a database for each selected strain having as target to include them in a collection of industrial microorganisms and to use for the study of biomolecules derived from them.

120 microbial strains were screened, isolated and identified including pigmented bacteria as Serratia marcescens pigmented by a reddish-orange tripyrrole called prodigiosin and Aureobasidium pullulans yeast "like mold", pigmented in pink to brown and black.

As conclusions, some isolated bacterial strains were inhibited by 10% of salt and isolated yeast strains were inhibited in very high percentage of sugars above 50%. Isolated yeast strains were more tolerant toward salt percentages, applied during experiments. Bacterial sporulation, pigmentation and pseudo micelle formation of yeasts were depended by specific conditions caused by sugar and salt content.

Key words: Liquid substrates, Osmophilic microorganisms, Morphological characteristics, Physiological characteristics.

1. Introduction

Pigmented microorganisms especially pigmented bacteria and yeasts are those who produce pigments with different colors as: orange, red, reddish, yellow, green, blue, black, depending also by the growth media. There are well known strains studied in details as producers of: carotenoids, phenazine derivatives, melanins and others. Morphological characterization of many colorful strains means that different genera and species have the property to produce chromophore substances. Some of them are distributed in the air and waters. Some are osmophilic and others have a high development in specific conditions of temperature and activity of water. Pigmented cocci and bacilli are studied and their pigments are determined in order to be analyzed chemically.

Some of the most important microbial pigments are: carotenoids, flavonoids, tetrapyrroles, xanthophylls as astaxanthin etc., studied also for their industrial importance in pharmaceutical biotechnology, in food technology, aquaculture etc. A large quantity of pigments are produced by Rhodotorula, Phaffia, Serratia, Acromobacter, Yarrowia and others. So, yellow riboflavins synthesized by some microbial strains as Candida famata and Bacillus subtilis are used in food technologies including baby foods and milk products. Carotenoids produced by Rhodotorula spp., Serratia spp. and...
2. Materials and Methods

A three year experimental research work focused in the isolation of the pigmented microorganisms was performed having as target the discovery of the colorful bacteria and yeasts preparing also the collection of pigmented microorganisms.

The sampling was focused principally in a coastal environment of Adriatic Sea about 40 km from Tirana. Isolation and identification of pigmented strains were made from selected samples of sea water and water supplies from the same environments. Liquid phases with high percentage of sugars were used also having as target the discovery of specific pigmented yeasts and molds. The sampling was performed during spring seasons when the seaside was not populated.

35 samples from water supply and 5 samples of sea water were taken from coastal environments of Adriatic Sea and were analyzed on the Laboratory of Microbiology in the Faculty of Natural Sciences - Tirana University. Marine samples were collected from surface waters of the three different experimental points in the same coastal environment. Water supply samples were collected from six experimental points of the seaside in the same period of the year.

Total microbial charge were determined for each sample, calculating also the percentage of pigmented microorganisms towards other explored strains. Plate Count Agar (PCA) and Potato Dextrose Agar (PDA) media were used for the determination of the total number of microorganisms. Screening of pigmented strains was made for all the samples using the same media cultures. Isolation and identification methods were applied in order to obtain purified representative colorful microorganisms. Taxonomic methods including those auxonographics, were used in order to make the identification of the obtained purified strains. Microbial growth and the resistance of the isolated strains towards changes of osmotic pressure were studied for some of the identified colonies. The behavior of natural and synthetic inhibitors - vibramycin, a synthetic antimicrobial and juices of blueberry and black cherries towards pigmented strains were studied in details using Disc Diffusion Method - DDM. Minimum Inhibitory Concentrations (MIC) determined before for each used antimicrobial (20 and 25 µl) were applied above Machery Nagel discs to observe reduction of the strains development.

120 microbial strains were obtained, 43% were red pigmented bacteria including those reddish with white borders and red pigmented yeasts, 27% black color strains, 28% black color strains, 1% green color strains and 1% blue color strains.

Reddish, yellow and beige color strains were used to study the effect of the above natural and synthetic antimicrobials.

3. Results and Discussion

The determination of the total microbial charge for the selected samples was performed by inoculating the water in PCA and PDA, by applying the method of limited dilutions. After the first inoculations and inhibitions, the small number of the developed colonies suggested a simple and direct inoculation of samples, without the application of the above method. The incubation was performed in 28 °C and 37 °C. The number of pathogens were negligible, so all the other incubations were performed only in the first temperature.

The total microbial charge for the most populated samples is presented in the following chart (Figure1) when the series 1 belongs to pigmented colonies and the series 2 to total charge.

Pigmented microorganisms were selected from samples above and all others and were purified in the same media culture PCA and PDA.

The percentage of pigmented colonies in the above samples and in some others with a small total charge number but with a considerable percentage of those pigmented is described in the chart of Figure 2. The samples codified as wspg-15 and wsp-14 have the highest percentage of the pigmented colonies.
A considerable number of pigment producers was selected in the samples of sea water taken from the surfaces in study and also from some selected liquid phases used, as syrups with a high percentage of glucose and other carbon sources. Chemical substances of the liquid phases-ingredients or additives induced the development of screened pigmented microorganisms.

The most populated were samples of sea water codified swg-14, swg-1-14, swp-3-14 and liquid phases codified pv-1-14, pv-2-14, mg-14, mg-15 (Figure 3).

Liquid syrups have small percentage of pigmented colonies, compared with the samples of sea surface water, but they are very special and resistant strains taking into consideration the inhibition tests.

The percentage of each microbial pigmented group is as follows (Figure 4).

Some selected samples, screened as representatives of each groups of pigmented strains, were developed in liquid media, rich with salt and sugars in order to observe their behavior in the conditions of high osmotic pressures. Two series of liquid media were prepared. The first one with 1%, 2%, 3%, 4% ... 30% of NaCl and the second with 5%, 10%, 15% ... 70% of sucrose.

Physiological tests gave a very interesting inhibition of strains in the values of about 10% of salt and about 50% of sugars. Results for some strains are introduced in the chart shown in Figure 5. Inhibition from salt percentages and sugar percentages are as follows:

Determined intervals of salt and sugar that have caused the reduction of the development and the diminution of fermentative properties (CO₂ production are respectively [5 - 10.5%] of NaCl and [40 - 55%] of sucrose. All *Rhodotorula* strains are inhibited in 10% of NaCl and 50% of sugars. In some cases the same strain isolated from different samples, developed in different media also, presented different values of inhibition. Colonies obtained by surface sea waters were most resistant toward increasing values of salt. Adriatic Sea has a salinity about 35 - 39 practical salinity units (PSU), so the appeared resistance of colorful strains is clear.
Identification methods were applied for principal selected strains. Explored genera and species are included in Table 1.

A dominance of *Rhodotorula* species was observed. *Aureobasidium phyllosphere* was also present in water supply from coastal environments. It is well known its adaptation in hyper saline environments. Environmental contaminants as bacilli were isolated and identified. *Sarcina* spp. were also microbial populations of some selected samples. It was the only strain well developed in 37°C. Colonies of *Rhodotorula mucilaginosa*, *Bacillus megatherium*, *Bacillus subtilis* and *Sarcina lutea* grown first in liquid media, were developed in PDA solid substrates and were treated with the synthetic antimicrobial - vibramycin and after with some fruit juices known and used as antimicrobials. The inhibition of the development associated with sporulation, reduction of color and inhibition of reproduction was strongly observed. Disc Diffusion Method was applied successfully for all the selected strains. The reduction of the development is presented in Figure 6. Inhibition effects are clear and in some cases an almost equal inhibition with synthetic antimicrobial vibramycin was achieved using a natural antimicrobial substance. The development of *Rhodotorula mucilaginosa* was well reduced together with the reduction of the chromophore pigments of cell membrane.

### 4. Conclusions

- Surface waters of Adriatic Sea are important sources for obtaining pigmented microorganisms. Other sources of pigmented bacteria and yeasts are water supply of coastal towns and also some pharmaceutical liquid forms, as syrups with high percentage of glucose and other sugars.
- Osmophylic pigmented microorganisms were isolated from above mentioned environments, determining also the percentage of the developed colonies compared with others non-pigmented.

**Table 1. Identified species of pigmented strains isolated from sea water, water supply and liquid phases with high percentage of carbon sources**

<table>
<thead>
<tr>
<th>Codified sample</th>
<th>Genius</th>
<th>Species</th>
<th>Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>swg-1-14</td>
<td><em>Rhodotorula</em></td>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>Carotenoid pigment</td>
</tr>
<tr>
<td>swg-4-12</td>
<td><em>Rhodotorula</em></td>
<td><em>Rhodotorula gracilis</em></td>
<td>Carotenoid pigment</td>
</tr>
<tr>
<td>swp-3-17</td>
<td><em>Rhodotorula</em></td>
<td><em>Rhodotorula glutinis</em></td>
<td>Carotenoid pigment</td>
</tr>
<tr>
<td>wsg-1-13</td>
<td><em>Serratia</em></td>
<td><em>Serratia marcescens</em></td>
<td>Reddish-orange tripyrrole pigment- prodigiosin</td>
</tr>
<tr>
<td>wsg-1-14</td>
<td><em>Aureobasidium</em></td>
<td><em>Aureobasidium pullulans (phyllosphere)</em></td>
<td>Melanin pigment and pullulan producer</td>
</tr>
<tr>
<td>wsg-2-14</td>
<td><em>Rhodotorula</em></td>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>Carotenoid pigment</td>
</tr>
<tr>
<td>wsp-17-15</td>
<td><em>Micrococcus</em></td>
<td><em>Micrococcus lutea (Sarcina lutea) isolated in 37°C</em></td>
<td>Xanthophylls substance</td>
</tr>
<tr>
<td>pv-5</td>
<td><em>Rhodotorula</em></td>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>Carotenoid pigment</td>
</tr>
<tr>
<td>mg-1</td>
<td><em>Bacillus</em></td>
<td><em>Bacillus megatherium</em></td>
<td>Brown pigment</td>
</tr>
<tr>
<td>mg-1</td>
<td><em>Bacillus</em></td>
<td><em>Bacillus subtilis</em></td>
<td>Brown pigment</td>
</tr>
<tr>
<td>mg-3</td>
<td><em>Bacillus</em></td>
<td><em>Bacillus alvei</em></td>
<td>Beige to brown pigment</td>
</tr>
</tbody>
</table>
- A dominance of *Rhodotorula* species was observed in all the analyzed samples, as *Rhodotorula mucilaginosa* and other similar species known and studied as carotenoid producers.

- Microbial resistance towards high percentages of salt and sugars were studied in order to determine the optimal concentrations of NaCl and sucrose in the inhibition of the development of isolated pigmented microorganisms. Optimal concentrations for a maximal inhibition were 10% of NaCl and 50% of sucrose.

- Inhibition was observed during morphological and physiological tests—number of developed vegetative cells, sporulation, pseudo micelle development, reduction of carotenoid pigments of *Rhodotorula* spp. and chromophore substances of other strains, fermentation and CO₂ production.

- Disk Diffusion Method was successfully performed with a large scale reduction of growth and cell reproduction. Antibiotic Vibramycin and blue berry juice were very effective as synthetic and natural antimicrobials. Their effective action is related with the reduction from 10% to 30% of the values for all tested colonies, using 25 µl as MIC. Blueberry solution was a stronger natural antimicrobial compared with black cherry juice.

5. References


